

Soluble Tumor Necrosis Factor (TNF) Receptors Are Effective Therapeutic Agents in Lethal Endotoxemia and Function Simultaneously as Both TNF Carriers and TNF Antagonists

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ABSTRACT. Two forms (monomeric or dimeric) of the extracellular, ligand-binding portion of the human p80 cell-surface receptor for TNF were used to antagonize TNF activity in vitro and in vivo. The dimeric sTNFR:Fc molecule was a more potent inhibitor of TNF than the monomeric sTNFR (50 to 1000 \times), as assessed in vitro by inhibition of TNF binding or bioactivity and in vivo by protection of mice from an otherwise lethal injection of LPS. Surprisingly, the dimeric sTNFR:Fc construct demonstrated a beneficial effect even when administered 3 h after a lethal LPS injection (i.e., after serum TNF levels had peaked and receded). To study the mechanism by which the soluble TNFR functions in vivo, serum TNF levels were examined in mice given LPS in the presence or absence of soluble receptor. Administration of a mortality-reducing dose of sTNFR:Fc ablated the rise in serum TNF bioactivity that normally occurs in response to LPS. However, TNF bioactivity was revealed in these "TNF-negative" serum samples when the L929 bioassay was modified by inclusion of a mAb that blocks the binding of murine TNF to the human soluble TNF receptor. These results indicate that the absence of direct cytolytic activity in the L929 assay was caused by neutralization of TNF, rather than to an absence of TNF in the serum. Moreover, administration of either monomeric sTNFR or low doses of dimeric sTNFR:Fc actually resulted in increased serum TNF levels compared to mice given LPS but no soluble receptor. However, these "agonistic" doses of soluble receptor did not lead to increased mortality when an LD₅₀ dose of LPS was given. Thus, dimeric sTNFR are effective inhibitors of TNF and under some circumstances function simultaneously as both TNF "carriers" and antagonists of TNF biologic activity. *Journal of Immunology*, 1993, 151: 1548.

TNF is a polypeptide hormone released by activated macrophages and T cells, which mediates a wide range of biologic functions. In addition to its potential role as a regulator of the normal immune response, TNF is also thought to play a major role in systemic toxicity associated with sepsis (1-6). TNF may also be involved in the pathogenesis of AIDS (7-9) as well as a number of autoimmune and inflammatory diseases (10-13). A mole-

cule that specifically inhibits the biologic activities of TNF may thus have considerable therapeutic utility.

Soluble, extracellular, ligand-binding portions of cytokine receptors occur naturally in body fluids and are believed to regulate the biologic activities of cytokines (14-17). The importance of these molecules as cytokine regulators is underscored by the fact that several pox viruses encode proteins with structural and functional homology to the extracellular portions of the receptors for TNF and IL-1 (18-20). Considerable controversy exists concerning the type of regulatory role naturally occurring soluble cytokine receptors might perform. Although it is likely that such molecules will function as cytokine carriers in an operational sense by altering the biodistribution of the cytokine to which they bind, it is not clear whether such an interaction would serve to agonize or antagonize the biologic

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effects of the cytokine (21, 22). However, experiments in which recombinant soluble receptors have been administered *in vivo* demonstrate their potential to inhibit immune and inflammatory responses, presumably by acting as antagonists of cytokine activity (23, 24).

There are two distinct cell-surface receptors for TNF: the 80 kDa (p80) and the 60 kDa (p60) receptors, both of which bind TNF- α and TNF- β (25, 26). Given the predominantly trimeric nature of TNF (25) and the apparent requirement for cross-linking of cell-surface TNFR for signal transduction (27), it is likely that dimeric soluble receptor constructs should possess a higher affinity for TNF (28) and therefore function as considerably more potent competitive inhibitors than monomeric sTNFR.² This prediction has been verified by the results of recent experiments demonstrating superior TNF inhibitory activity of dimeric Fc fusion constructs of p60 *in vitro* (29). Although soluble forms of both monomeric and dimeric p60 TNFR have been shown to be beneficial in animal models of sepsis, no direct comparison of the *in vivo* potency of monomeric vs dimeric receptors in sepsis has been reported. In addition, little information is available concerning the mode of action of such inhibitors *in vivo*.

Monomeric and dimeric (Fc fusion protein) forms of the p80 TNFR were constructed and compared *in vitro* and *in vivo* for effects on TNF biologic activity. The results indicate that the sTNFR:Fc, but not the sTNFR, was effective in reducing mortality associated with LPS administration, at least over the concentration range tested. In addition, the sTNFR:Fc molecule can function simultaneously as both a TNF "carrier" and an antagonist of TNF biologic activity and thus inhibit the lethal effects of LPS by acting as a biologic buffer for TNF.

Materials and Methods

Mice

BALB/c female mice 8 to 10 wk old were purchased from Charles River (Wilmington, MA) and were maintained within a specific pathogen-free environment.

Construction and production of p80 sTNFR and sTNFR:Fc

Recombinant sTNFR was expressed in a CHO cell line using the glutamine synthetase selectable and amplifiable marker. For production, cells cultured to confluence in roller bottles were washed with PBS and then cultured in serum-free medium. Purification of the sTNFR from the CHO supernatant was accomplished in a single affinity

chromatography step using a mAb, M1, specific for sTNFR.

Recombinant sTNFR:Fc was expressed in CHO cells using the dihydrofolate reductase selectable and amplifiable marker. Suspension cells were centrifuged and resuspended into serum-free medium in a controlled bioreactor. The product was collected after 7 days. The sTNFR:Fc molecule was purified using protein A affinity chromatography followed by an ion-exchange step.

Concentrations of the purified sTNFR and sTNFR:Fc were determined by amino acid analysis. Endotoxin levels were determined to be <5.6 ng endotoxin/mg sTNFR or sTNFR:Fc using the Kinetic-QCL assay (Whittaker Bioproducts, Walkersville, MD) for detection of Gram-negative bacterial endotoxin. Physical characterization included SDS-PAGE, N-terminal sequencing, and immunoreactivity analyses (K. E. Stramler and H. Madani, unpublished observations). A diagrammatic representation of p80 sTNFR and sTNFR:Fc is shown in Figure 1.

Antibodies to soluble TNFR

The generation of mAb to the human p80 sTNFR has been described previously (30). M1 mAb (rat IgG 2b) and M3 (rat IgG) mAb both bind to the human p80 sTNFR but not to mouse TNFR.

Binding inhibition assay

Human rTNF- α was expressed in yeast as a protein composed of the entire coding region of mature TNF fused to an octapeptide at the N terminus, useful in affinity purification. Purified TNF was radioiodinated as described (18) to a sp. act. of 2×10^{15} cpm/mmol, without loss of biologic activity (measured in an L929 cytotoxicity assay) or receptor-binding activity (see below).

Inhibition assays were carried out as described (31). Briefly, [¹²⁵I]TNF- α (0.5 nM) was incubated in binding medium (RPMI 1640, 2.5% BSA, 50 mM HEPES buffer, pH 7.4, 0.4% NaN₃) for 2 h at 4°C with serially diluted inhibitors (human sTNFR:Fc, sTNFR monomer, or unlabeled human rTNF- α) and 2×10^6 U937 cells. Duplicate aliquots were subsequently removed, centrifuged through a phthalate oil mixture to separate free and bound ligand, and the radioactivity was measured on a gamma counter. Nonspecific binding values were determined by inclusion of a 200 \times molar excess of unlabeled TNF and were subtracted from total binding data to yield specific binding values. Data were plotted and results analyzed as described (31).

L929 bioassay for TNF activity

The protocol used to measure the presence of TNF cytolytic activity using L929 cells as targets has been described previously (32, 33). Briefly, 10 μ l of mouse serum, mouse

² Abbreviations used in this paper: sTNFR, soluble monomeric human p80 TNFR; sTNFR:Fc, recombinant fusion protein composed of soluble dimeric human p60 TNFR linked to the Fc region of human IgG1; CHO, Chinese hamster ovary.

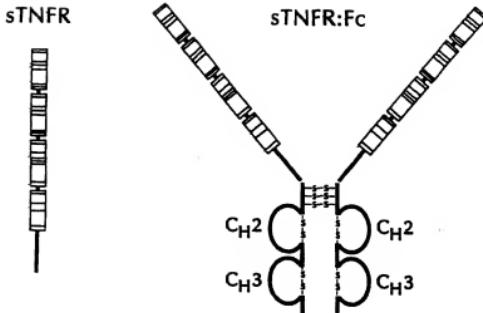


FIGURE 1. Construction of monomeric sTNFR and dimeric sTNFR:Fc molecules. Extracellular portions of the human p80 TNFR cDNA were cloned and produced as described in *Materials and Methods*. In the dimeric sTNFR:Fc molecule three disulfide bonds are depicted. However, the disulfide bond closest to the N terminus is normally used for binding to the Ig L chain and thus, its state (i.e., free cysteine or disulfide bond) in the sTNFR:Fc fusion product is not known.

rTNF- α (Genzyme, Boston, MA), or supernatant from LPS-stimulated RAW 264.7 cells (American Type Culture Collection, Rockville, MD) was serially diluted (50%:50%, v/v) in flat bottom, 96-well microtiter plates. L929 medium (RPMI 1640 with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin) was added to each well, followed by soluble receptors, control proteins, or mAb in a total volume of 30 μ l. Ten microliters of actinomycin D was then added (final concentration of 0.1 μ g/well; Sigma, St. Louis, MO). Finally, 5 \times 10⁴ L929 cells were added to each well (final volume/well = 100 μ l) and the plates were incubated at 37°C in 5% CO₂. To prevent the influence of edge effects on the TNF bioassay, only the inner wells of each plate were utilized. All outer wells received 200 μ l of L929 medium only. After 16 h of incubation, the culture medium was removed and replaced with 200 μ l of 0.5% crystal violet in methanol/water (1/4). The plate was washed with distilled water and air dried at ambient temperature. One hundred microliters of 2% deoxycholic acid (catalog no. D-6750, Sigma) was added to each well to solubilize the crystal violet and the plates were analyzed on an ELISA plate reader at 562 nm. The negative control consisted of L929 cells in the presence of actinomycin-D. Estimates of serum TNF concentrations were obtained by comparing the TNF activity in the experimental serum samples with the activity obtained with the mouse rTNF- α standard.

LPS-induced mortality

LPS, derived from *Escherichia coli* 0127:B8 (catalog no. DF3132-25, VWR, Seattle, WA), was resuspended at 10 mg/ml in sterile saline and stored at -20°C in small aliquots. The LPS was diluted to the proper concentration and sonicated (CU-6 sonicator; Branson, Shelton, CT) for 1 min before injection. BALB/c female mice (18 to 20 g) were injected i.v. with an LD₆₀ to LD₁₀₀ dose of LPS (300 to 400

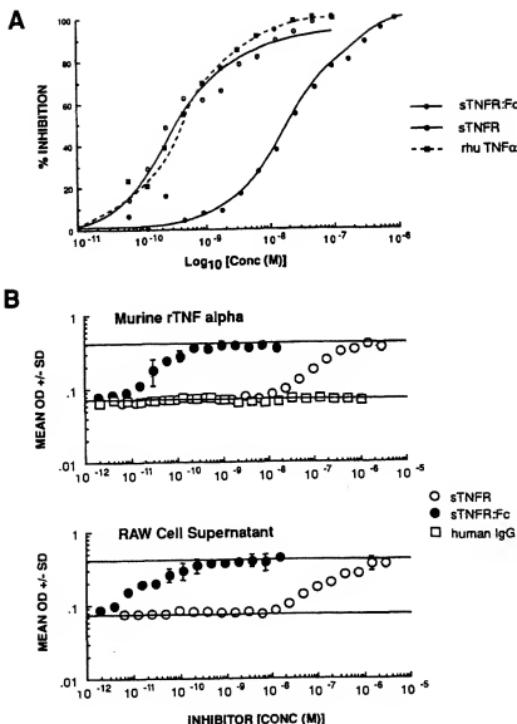
μ g) in 0.2 ml of saline. The LPS was injected either alone or in conjunction with sTNFR, sTNFR:Fc or control protein, human IgG (catalog no. I-4506, Sigma). In some experiments, mice were injected with LPS i.v. followed at 2, 3, or 4 h with an i.v. injection of soluble receptor or control protein. Survival was monitored for at least 5 days and, in some experiments, the mice were observed for a maximum of 4 wk. However, no further mortality occurred after the initial 5-day observation period.

Results

In vitro neutralization of TNF activity by soluble TNFR

The ligand binding characteristics of sTNFR monomer and sTNFR:Fc were determined by cell-based inhibition studies using ¹²⁵I-human rTNF- α and U937 cells expressing surface p80 and p60 TNFR. Results of these experiments are shown in Figure 2A. To generate a robust criterion of the relative activity of the sTNFR:Fc, we have analyzed the binding inhibition data with a simple one-site model to yield a single K_i , which reflects that concentration of inhibitor which mediates 50% inhibition of binding of TNF to cell-surface receptors. As predicted from (1) the multi-valent interactions that occur between TNF ligands and receptors and (2) previous studies (29), the sTNFR:Fc ($K_i = 1 \times 10^{10} \text{ M}^{-1}$) shows ~50-fold higher affinity for the ligand than does the sTNFR monomer ($K_i = 2 \times 10^8 \text{ M}^{-1}$). Thus, one might suspect that the sTNFR:Fc molecule would be a better antagonist of TNF biologic activity in comparison to the monomeric sTNFR *in vitro* and *in vivo*. To address the biologic efficacy of monomeric (sTNFR) and dimeric (sTNFR:Fc) forms of the soluble p80 TNFR, both molecules were analyzed for their ability to neutralize TNF activity *in vitro* in the L929 bioassay (Fig. 2B). Monomeric sTNFR and dimeric sTNFR:Fc inhibited the ac-

FIGURE 2. Comparison of TNF binding and neutralizing capability of sTNFR and sTNFR:Fc. *A*, U937 cells (2×10^6) were incubated at 4°C for 4 h with 0.5 nM [125 I]-human rTNF- α in binding medium and varying concentrations of inhibitor (sTNFR:Fc, sTNFR monomer, or unlabeled human rTNF- α) in a total volume of 150 μ l. Duplicate 70- μ l aliquots of the suspension were subsequently removed and microfuged through a phthalate oil mixture to separate free and bound ligand. Radioactivity was measured in a gamma counter and the data were analyzed according to a simple competitive inhibition model. *B*, a constant amount of murine rTNF- α (125 pg/ml) or natural TNF (derived from LPS-stimulated RAW cells, 1/200 dilution) was added to each well of an L929 cytotoxicity assay in the presence of varying amounts of inhibitors (sTNFR, sTNFR:Fc or human IgG). Details of the L929 cytotoxicity assay are provided in *Materials and Methods*. The OD of L929 cells in the absence of TNF is indicated by the *upper solid line* (mean OD approximately 0.45) and maximal lysis of L929 cells is indicated by the *lower solid line* (mean OD approximately 0.075).



tivity of mouse TNF (recombinant or natural) in a dose-dependent fashion; however, sTNFR:Fc was approximately 1000-fold more efficient than sTNFR. Identical results were obtained when human rTNF- α was utilized as the ligand (data not shown). Human IgG, used as a control protein, had no effect on TNF activity.

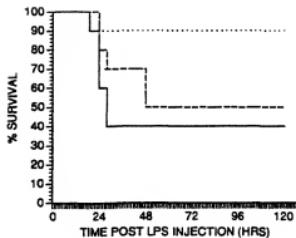
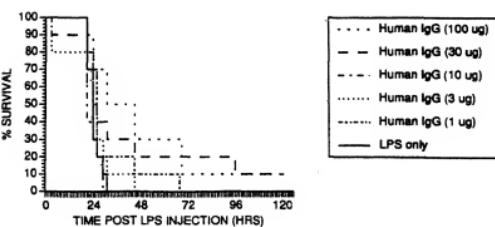
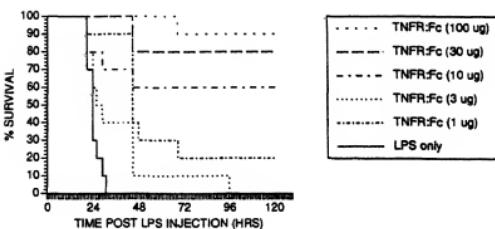
Ability of sTNFR to prevent mortality induced by LPS

We have also compared the biologic efficacy of sTNFR and sTNFR:Fc *in vivo* in a murine model of LPS-induced septic shock. Various doses of sTNFR:Fc or control protein (human IgG) were mixed with a lethal dose of *E. coli* LPS (400 μ g/mouse) and injected i.v. into 18- to 20-g BALB/c female mice. Survival was monitored for 5 days and the results are presented in Figure 3. Treatment of mice with LPS only or LPS and any dose of human IgG resulted in 0 to 10% long

term survival. In contrast, 90% of mice treated with LPS plus 100 μ g (1.95 nmol) of sTNFR:Fc survived. Beneficial effects of the sTNFR:Fc protein were also evident with doses as low as 10 μ g (0.2 nmol)/mouse. In similar studies we have been unable to demonstrate an effect of recombinant monomeric sTNFR on survival even when doses as high as 260 μ g (10.35 nmol) were administered (Fig. 4). However, based on the *in vitro* neutralizing capacity of the monomeric vs dimeric sTNFR (Fig. 2) and the dose of sTNFR:Fc required to effect survival *in vivo* (Fig. 3), monomeric sTNFR would be predicted to demonstrate efficacy at much higher doses (10 mg/mouse).

The ability of the sTNFR:Fc protein to provide protection when given at various times after LPS administration was also tested. Mice received a lethal dose of LPS (i.v.) followed 2, 3, or 4 h later by sTNFR:Fc (100 μ g/mouse). Two to three separate experiments were conducted for each

FIGURE 3. Administration of sTNFR:Fc prevents mortality of BALB/c mice injected with a lethal dose of LPS. Various doses of sTNFR:Fc or human IgG, as a control, were mixed with a lethal dose of LPS (400 μ g) and injected i.v. into BALB/c mice. Survival was monitored at least once a day for 5 days. In each of three separate experiments, mice treated with sTNFR:Fc at doses of 10 μ g or above demonstrated enhanced survival.



time point. All experiments provided similar results and therefore the results were pooled (Fig. 5). The results demonstrate that the administration of sTNFR:Fc was clearly beneficial even when administered up to 3 h after the injection of LPS. In the same experiment, the progression of serum TNF activity after LPS injection was determined in a subset of mice that received LPS only (Fig. 6). These experiments and previous reports (34-36) demonstrate that most of the serum TNF activity was produced during the first 2 h after LPS administration. These results demonstrate that the sTNFR:Fc protein was efficacious even when administered after serum TNF levels had peaked and receded. Thus, the efficacy of the sTNFR:Fc molecule must not be due solely to neutralization of serum TNF bioactivity.

Effect of sTNFR and sTNFR:Fc on serum TNF levels in vivo

To study the mechanism by which sTNFR:Fc protected mice from an otherwise lethal dose of LPS, the effect of the two forms of soluble TNFR on TNF activity present in the serum was examined. Mice were injected with LPS alone (400 μ g) or LPS mixed with 100 μ g of sTNFR, sTNFR:Fc, or control protein, human IgG. Serum samples were obtained 2 h after injection and assayed for TNF bioactivity (Fig. 7). Mice injected with LPS alone or LPS mixed with human IgG exhibited equivalent amounts of serum TNF activity (approximately 1 ng/ml) 2 h after LPS injection. In contrast, mice treated with LPS plus 100 μ g of sTNFR:Fc

FIGURE 4. Administration of sTNFR does not affect mortality of BALB/c mice injected with a lethal dose of LPS. The procedure was identical to that described in the legend to Figure 3. Note: the response of mice treated with human IgG plus LPS overlaps the response of mice treated with sTNFR (260 μ g) plus LPS.

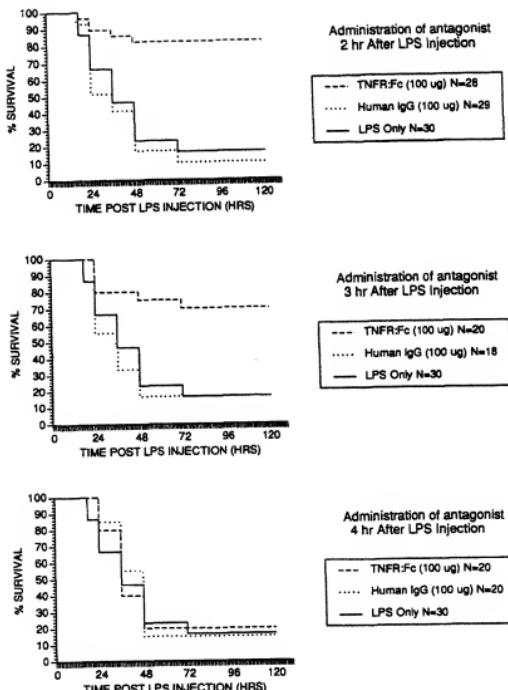


FIGURE 5. Administration of sTNFR:Fc prevents mortality of BALB/c mice even when injected 3 h after a lethal dose of LPS. At 2, 3, or 4 h after i.v. injection of a lethal dose of LPS (400 µg), 100 µg of sTNFR:Fc or human IgG, as a control, were injected i.v. Survival was monitored at least once a day for 5 days. The results represent a compilation of two to three separate experiments.

(which protects mice from the lethal effects of LPS injection, (Fig. 3)) had little or no serum TNF activity as assessed in the L929 assay. Somewhat surprisingly, mice treated with an equivalent dose of monomeric sTNFR (which was not efficacious in survival studies) exhibited serum TNF levels 10-fold higher (10 ng/ml) than control mice treated with LPS only or LPS plus human IgG.

Figure 8 depicts results of an experiment in which the relationship between the dose of sTNFR:Fc and serum TNF activity was examined. Sera obtained from mice injected with LPS alone or LPS plus 1 to 100 µg of human IgG contained detectable TNF activity that titrated in a predictable fashion. Sera obtained from mice 2 h after treatment with 100 or 30 µg of sTNFR:Fc and LPS contained little if any demonstrable TNF activity. Mice injected with 10, 3, or 1 µg of sTNFR:Fc and LPS exhibited serum TNF activity but the sera displayed unusual characteristics. These serum samples demonstrated intermediate levels of

TNF activity, which failed to decrease even when diluted to 1/160 (Fig. 8) (data not shown). Because these results were obtained only when mice received LPS and low doses of the sTNFR:Fc, we examined the influence of the sTNFR:Fc on TNF activity in these samples.

Ability of sTNFR:Fc molecules to act as carriers of TNF

Experiments were conducted to determine the effect of blocking the TNF-binding ability of sTNFR:Fc molecules *in vitro* in the L929 cytotoxicity assay. To this end, we utilized a mAb (M1) that binds to the sTNFR:Fc molecule and blocks the ability of the soluble human TNFR:Fc protein to bind TNF. Another rat mAb (M3) that binds the sTNFR:Fc molecule but does not block TNF binding was used as a control. To examine the ability of M1 to block TNF binding to sTNFR:Fc proteins, constant amounts of sTNFR:Fc (200

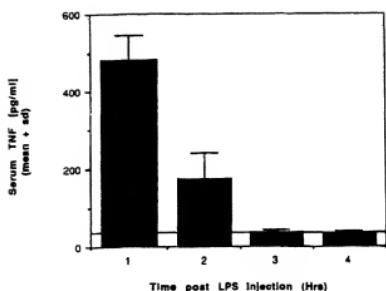


FIGURE 6. Serum TNF levels are elevated for 2 h after lethal LPS injection. Mice were injected with a lethal dose of LPS (400 μ g) and serum was obtained at 1, 2, 3, or 4 h. TNF activity was assessed by the L929 cytolysis assay as described in *Materials and Methods*.

ng/ml) and murine rTNF α (125 pg/ml) were added to dilutions of M1, M3, or rat IgG (Fig. 9). The ability of the sTNFR:Fc protein to neutralize the activity of TNF was reversed only in the presence of M1. In addition, full neutralization of the sTNFR:Fc protein (200 ng/ml) required a 10-fold excess (2 μ g/ml) of M1.

The effect of addition of M1 to serum obtained from mice 2 h after injection of LPS (400 μ g) mixed with 10 or 100 μ g of sTNFR:Fc was examined. As previously described (Fig. 8), the serum obtained from mice treated with 10 μ g of sTNFR:Fc demonstrated intermediate levels of activity that were not altered by dilution (Fig. 10). Addition of M1 (2 μ g/ml) to dilutions of the serum revealed the presence of additional TNF activity, which titrated in a predictable fashion. As expected, addition of control antibody (i.e., M3 or rat IgG) had no effect on the TNF activity. Furthermore, addition of M1, M3, or rat IgG had no effect on serum samples that did not contain the soluble human TNFR:Fc protein (i.e., sera obtained from mice injected with LPS and human IgG), demonstrating that the antibody did not affect the ability of mouse TNF to bind to the indicator L929 cells (Fig. 10). We have also examined serum samples from mice treated with a higher dose of sTNFR:Fc (100 μ g) and LPS. In the absence of manipulation these samples did not demonstrate TNF activity in vitro. However, TNF activity was revealed when serum from these mice was treated with M1 but not with M3 or rat IgG (Fig. 10). In fact, maximal activity in the L929 assay of the sera from mice injected with sTNFR:Fc (100 μ g) and LPS was still apparent at serum dilutions of 1/100, whereas sera obtained from mice treated with LPS only or LPS plus human IgG demonstrated only small amounts of TNF activity at a dilution of 1/16 (Fig. 10).

To determine whether or not sTNFR:Fc could prolong the presence of serum TNF, mice were injected with LPS and 10 or 100 μ g of sTNFR:Fc or human IgG, as described above, and serum samples were obtained at 4 h. The serum samples were assayed in the L929 bioassay in the presence and absence of M1, M3, or rat IgG (Fig. 11). As expected, sera obtained from mice injected 4 h previously with LPS alone or LPS plus human IgG did not contain serum TNF activity. However, sera obtained from mice injected with LPS plus sTNFR:Fc (10 or 100 μ g) still contained biologically active TNF, which titrated in a predictable fashion in the presence of M1 mAb. Thus, mice injected with LPS and the soluble human TNFR:Fc protein, even at therapeutic doses, retained increased levels of TNF in the serum that persisted for longer periods of time. However, depending upon the dose of sTNFR:Fc administered, the TNF activity was either (1) enhanced or (2) revealed only upon the addition of a mAb which blocked the binding of TNF to the sTNFR:Fc protein. These observations indicate that the binding of the sTNFR:Fc protein to TNF is reversible and that the inhibition of TNF activity reflects a balance between the presence of sTNFR:Fc, TNF, and endogenous TNFR (either cell surface or soluble).

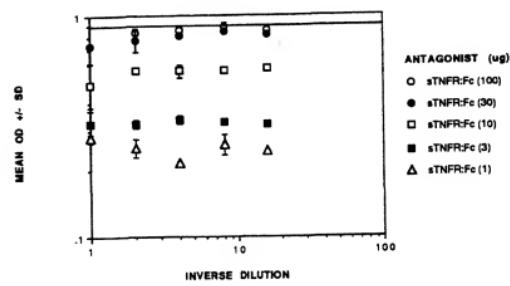
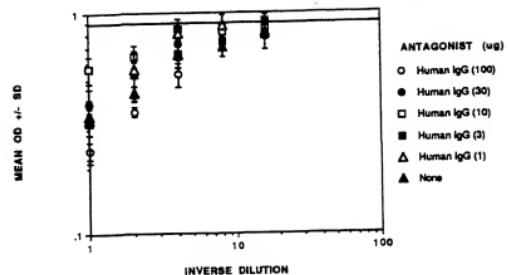
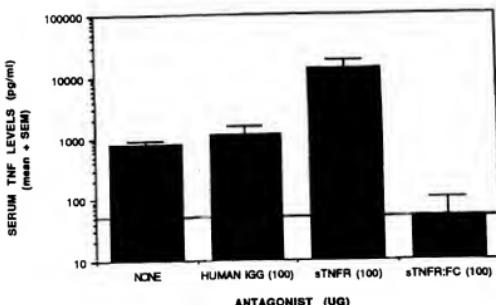
The carrier function of sTNFR:Fc molecules is not detrimental to the host

As the administration of sTNFR:Fc under some circumstances produced increased levels of serum TNF (Fig. 8) that persisted for at least 4 h (Fig. 11), it was important to determine whether or not the administration of sTNFR:Fc molecules under these circumstances would lead to detrimental consequences. Mice were injected with a dose of LPS (300 μ g) which produced intermediate levels of mortality (60 to 70%), such that beneficial or deleterious effects of the TNFR could be observed. Mice treated with sTNFR:Fc at doses ranging from 10 ng to 10 μ g demonstrated equivalent or slightly better survival when compared with mice treated with LPS alone or LPS and human IgG (Fig. 12). Further experiments in which lower doses of sTNFR:Fc (100 pg to 1 μ g) were utilized yielded similar results (data not shown). Thus, administration of sTNFR:Fc in sublethal models of LPS toxicity had no detrimental consequences on the survival incidence.

Discussion

The data presented in this report demonstrate that a fusion molecule consisting of a soluble form of the extracellular portion of the p80 cell surface TNFR fused to the Fc portion of human IgG1 (sTNFR:Fc) is an effective antagonist of LPS-induced septic shock. An increased incidence of survival in mice given an otherwise lethal dose of LPS was observed when the sTNFR:Fc protein was injected 0 to 3

FIGURE 7. The effect of sTNFR vs sTNF:Fc on serum TNF levels after co-administration of LPS in vivo. LPS (400 μ g) was mixed with 100 μ g of sTNFR, sTNF:Fc, or human IgG and administered i.v. to BALB/c mice. Serum samples were obtained 2 h after injection and analyzed for TNF activity in the L929 cytotoxicity assay. The results were obtained from three to four separate experiments for each treatment group. The sensitivity of the TNF bioassay is approximately 50 pg/ml and is indicated by the solid line.



h after LPS administration (Figs. 3 and 5). When administered simultaneously with LPS, doses of sTNF:Fc as low as 10 μ g (0.2 nmol)/mouse were beneficial (Fig. 3). In contrast, administration of up to 260 μ g (10.35 nmol) of the monomeric sTNFR failed to affect the incidence of mortality induced by LPS, even when the incidence of mortality in the control group was only 50% (Fig. 4). This difference

FIGURE 8. Analysis of TNF bioactivity in serum samples obtained 2 h after in vivo co-injection of LPS and sTNF:Fc. A lethal dose of LPS (400 μ g) was mixed with varying doses of sTNF:Fc or human IgG and injected i.v. into BALB/c mice. Serum was obtained from three mice in each group 2 h after injection. The serum for each group was pooled and analyzed for TNF activity in the L929 assay.

in efficacy between sTNF:Fc and sTNFR in vivo may be explained in large part by the higher affinity of TNF for sTNF:Fc than sTNFR, which results in a substantially greater ability of sTNF:Fc to neutralize the biologic effects of TNF (Fig. 2). Furthermore, linkage of the sTNFR to the Fc region of Ig imparts a fivefold longer serum $t_{1/2}$ to the sTNF:Fc molecule after i.v. injection (37), a property

FIGURE 9. Inhibition of the TNF neutralizing capacity of the human p80 sTNFR:Fc molecule by M1 but not M3 mAb. Dilutions of M1, M3, or rat IgG were added to constant amounts of sTNFR:Fc and murine rTNF- α in the L929 assay as described in *Materials and Methods*.

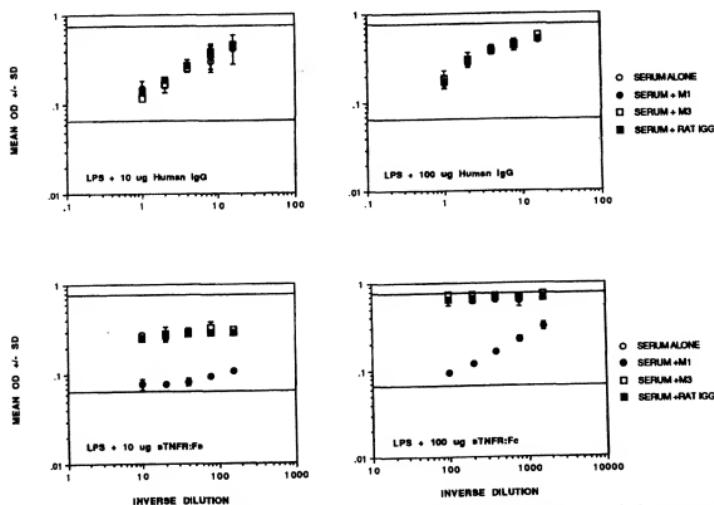
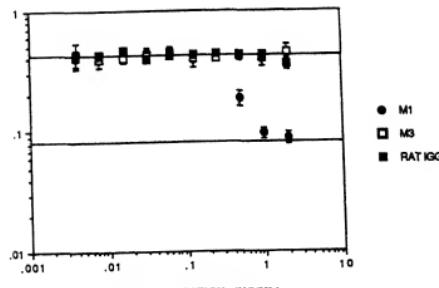


FIGURE 10. Demonstration of serum TNF activity in vitro in the L929 assay in the presence of M1 mAb. Serum was obtained from mice 2 h after injection of LPS (400 μ g) mixed with sTNFR:Fc or human IgG. The serum samples were serially diluted and assayed in the L929 cytotoxicity assay either alone or in the presence of constant amounts (2 μ g/ml) of M1, M3, or rat IgG.

that could also influence its efficacy in vivo.

The ability of sTNFR to alter the magnitude and time course of serum TNF after co-administration with LPS in vivo was examined. Sera from mice that received high, life-saving doses of sTNFR:Fc (e.g., 100 μ g) failed to exhibit significant levels of TNF bioactivity when assayed directly in the L929 cytotoxicity assay. However, further experimentation demonstrated that TNF was present in the serum but it was biologically inactive because of the con-

comitant presence of sTNFR:Fc. TNF activity in these samples was revealed in the presence of a mAb which blocked the ability of the human sTNFR:Fc molecules to bind TNF but did not interfere with the ability of TNF to bind to the murine TNFR on the surface of the L929 indicator cells (Figs. 9 to 11). These results suggest that the sTNFR:Fc protein has a relatively high exchange rate for TNF, such that once TNF is released in vitro, it can be detected if the TNF is inhibited from subsequently binding to free

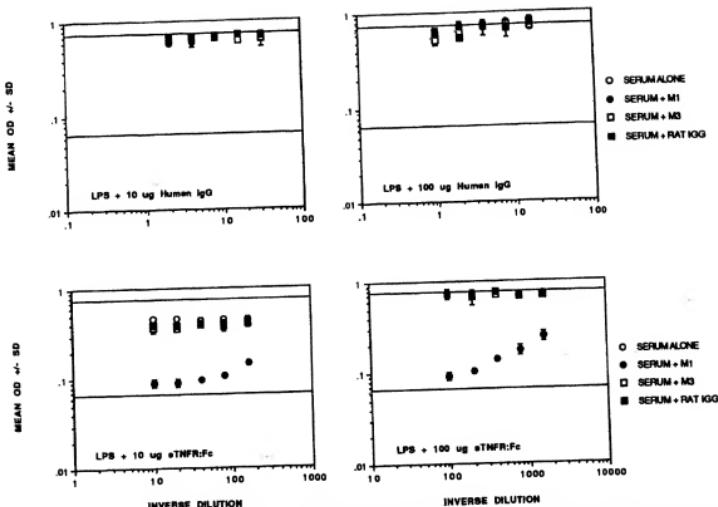


FIGURE 11. Prolongation of serum TNF in vivo by sTNFR:Fc. The protocol was identical to that described in the legend to Figure 10 except that the serum samples were obtained 4 h after LPS injection.

sTNFR:Fc molecules. If TNF is also released from the sTNFR:Fc molecule in vivo, the sTNFR:Fc molecules may function by dissipating the peak in serum TNF levels normally associated with bolus LPS injection.

Soluble TNFR:Fc molecules also function as carriers of TNF in that they alter the rate at which TNF disappears from the serum of LPS-treated mice. Control mice injected with LPS alone or LPS and human IgG had elevated serum TNF levels only during the first 2 h after injection (Figs. 6, 10, 11). However, mice treated with LPS and sTNFR:Fc retained TNF in their serum for at least 4 h (Fig. 11). In support of these data, we have demonstrated that the $t_{1/2}\beta$ of labeled TNF is increased approximately fourfold in vivo when injected concomitantly with sTNFR:Fc (D. Lynch and K. M. Mohler, unpublished observations). These results suggest that the sTNFR:Fc protein functions as an effective antagonist of LPS induced mortality by acting as a biologic buffer for TNF activity.

When mice were exposed to lethal doses of LPS and low doses of sTNFR, which failed to affect mortality incidence, serum TNF levels as detected in the L929 bioassay were elevated in comparison to control mice receiving LPS alone or LPS plus IgG (Figs. 7 and 8). However, despite the fact that low doses of sTNFR increased serum TNF activity, no agonistic activity in terms of mortality could be demonstrated when low doses of sTNFR were administered in

conjunction with an LD₅₀ dose of LPS (Fig. 12) (data not shown). These data indicate that the agonistic effects on serum TNF activity obtained in vivo in the presence of sTNFR were distinct from the effects of sTNFR on LPS-induced mortality. Alternatively, the sTNFR may function as an agonist only with lethal doses of LPS. If the latter hypothesis is correct, then lower (nonlethal) doses of LPS may induce sufficient quantities of endogenous soluble TNFR so that the administration of exogenous sTNFR:Fc molecules would have relatively minor additional biologic impact.

Several types of TNFR/antibody-based fusion proteins have been described and tested for efficacy in murine LPS-induced mortality models (38, 39). These TNF antagonists include the molecule employed in the present study, composed of the extracellular portion of the p80 cell-surface receptor linked to the Fc region of human IgG1, as well as molecules consisting of fusions between the extracellular portion of the p60 TNFR combined with the Fc region of either human IgG1 (38) or human IgG3 (39). The dose of p60 sTNFR:Fc (4 to 20 µg) (38, 39) and the dose of p80 sTNFR:Fc (10 to 100 µg) (Fig. 3) required to demonstrate efficacy are similar. However, efficacy of the different constructs was influenced substantially by the timing of administration relative to lethal LPS injection. The p80 sTNFR:Fc (human IgG1) construct was efficacious when

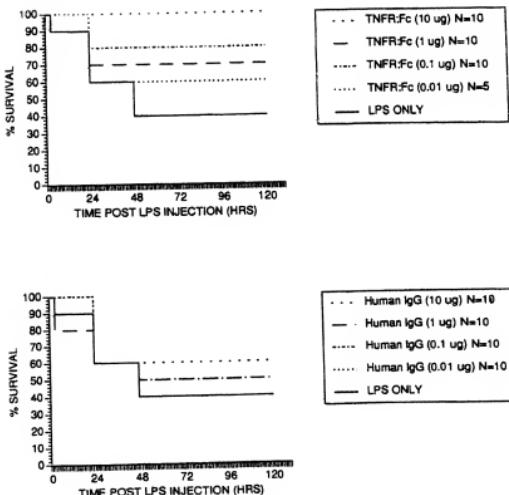


FIGURE 12. Administration of low doses of sTNFR:Fc is not detrimental to the host. BALB/c mice received an LD_{50} dose of LPS (300 μ g) premixed with low doses of sTNFR:Fc or human IgG. Survival was monitored at least once a day for 5 days.

administered as late as 3 h after LPS administration (Fig. 5). However, a sTNFR:Fc fusion protein consisting of the p60 sTNFR coupled to human IgG1 was effective only if administered within 1 h after lethal LPS injection (38). In contrast, preliminary reports utilizing the p60 sTNFR coupled with human IgG3 indicated that partial benefits were obtained as late as 3 h after LPS injection (39). Thus, significant differences exist between the published abilities of different sTNFR:Fc fusion proteins to function after LPS administration, and these differences do not appear to correlate with either the particular sTNFR (i.e., p60 or p80) or with the subclass of human IgG utilized for the fusion protein.

The relationship between serum TNF activity and efficacy of the sTNFR:Fc molecule had not been established before the present study. Given the ability of sTNFR:Fc to function effectively when administered as late as 3 h after LPS injection (Fig. 5), it was somewhat surprising to observe that the vast majority of detectable serum TNF activity had already passed by 3 h (Fig. 6). A number of hypotheses, which are not necessarily mutually exclusive, may explain these results. First, the length of time that TNF must be bound to its cell surface receptor prior to the induction of an irreversible biological effect such as cell lysis is unknown. However, studies by Engelberts et al. (40) suggest that TNF must be present for extended periods of time to achieve maximal biologic activity in vitro. Thus, sTNFR:Fc may be able to compete for TNF which has

already bound to the cell surface and, in effect, dislodge it before the interaction has occurred for a time sufficient to result in complete biologic signaling. In this regard, the rate of dissociation of radiolabeled TNF from its cell surface receptor in vitro is increased in the presence of either unlabeled TNF (41) or the dimeric sTNFR:Fc (C. Smith, unpublished results). Second, LPS-induced mortality may result from the cumulative effect of TNF. Thus, inhibition of the small amount of TNF present late in the time course might be sufficient to prevent mortality. Third, the therapeutic potential of the sTNFR:Fc molecule may not be related solely to the removal of serum TNF activity. The sTNFR:Fc molecule could function by inhibiting TNF activity in extravascular sites. Finally, LPS-induced toxicity may be mediated at least in part by TNF expressed on the cell surface, which may be masked in the presence of sTNFR:Fc. Regardless of the mechanism of efficacy of the sTNFR:Fc molecule, there is a relatively small window of time, 3 to 4 h after LPS injection, during which serum TNF levels are low and administration of the sTNFR:Fc molecule is still efficacious. These results also suggest that serum TNF levels may not always be a good prognostic indicator for the clinical efficacy of the sTNFR:Fc molecule.

Soluble TNF-binding proteins have been recovered from the urine of normal humans (42, 43) and appear at elevated levels in the serum of cancer patients (44, 45) and in response to endotoxin challenge (46). The biologic role of these TNF-binding proteins is currently under investiga-

tion. Previous investigators have demonstrated that TNF spontaneously loses activity in vitro and, under some circumstances, soluble p60 and p80 TNFR can prevent its spontaneous degradation, thereby enhancing the biological longevity of TNF (47). Our experiments demonstrated that a sTNFR monomer can function as an agonist of serum TNF activity in vivo and a sTNFR:Fc molecule could act either as an agonist or antagonist of serum TNF levels in a dose dependent fashion. Thus, the biologic effect of the soluble TNF-binding proteins isolated from humans will probably vary depending upon the relative concentration of TNF and sTNFR. This concept is supported by recent data of Girardin et al. (48), demonstrating increased concentrations of both TNF and soluble TNFR in the serum of septic patients. In that study, higher ratios of soluble TNFR to TNF correlated with increased probability of survival.

These experiments indicate that the sTNFR:Fc molecule is an effective antagonist of LPS-induced septic shock and are in agreement with a number of studies that have shown the beneficial effects of antagonizing TNF activity in sepsis with either antibody (1-3) or soluble receptors (38, 39). In aggregate, these results indicate that TNF plays a central role in mediating the lethality associated with sepsis. However, several lines of evidence suggest that the role of cytokines in sepsis is not yet fully understood. First, antagonism of several cytokines other than TNF (e.g., IFN- γ and IL-1) can also lead to beneficial results (49, 50). Second, anti-TNF antibodies have been reported to have variable therapeutic potential in models of endotoxemia, cecal ligation and puncture, and bacterial sepsis (51-53). Further experimentation will be required to determine whether or not the sTNFR:Fc molecule also displays the same spectrum of efficacy. However, the results presented here suggest that the sTNFR:Fc molecule may be a useful therapeutic agent for sepsis and other inflammatory diseases.

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